USE OF THE COMET ASSAY TO ASSESS GENOTOXICITY IN MAMMALIAN, AVIAN, AND AMPHIBIAN SPECIES

L.D. Knopper

National Wildlife Research Centre 2005
Canadian Wildlife Service
Environmental Conservation Branch

Technical Report Series Number 429

Environment Canada | Environnement Canada
Canadian Wildlife Service | Service canadien de la faune

Canada

106-03210/

TECHNICAL REPORT SERIES
CANADIAN WILDLIFE SERVICE

This series of reports, established in 1986, contains technical and scientific information from projects of the Canadian Wildlife Service. The reports are intended to make available material that either is of interest to a limited audience or is too extensive to be accommodated in scientific journals or in existing CWS series.

Demand for these Technical Reports is usually confined to specialists in the fields concerned. Consequently, they are produced regionally and in small quantities; they can be obtained only from the address given on the back of the title page. However, they are numbered nationally. The recommended citation appears on the title page.

Technical Reports are available in CWS libraries and are listed in the catalogue of the National Library of Canada in scientific libraries across Canada. They are printed in the official language chosen by the author to meet the language preference of the likely audience, with a résumé in the second official language. **To determine whether there is significant demand for making the reports available in the second official language, CWS invites users to specify their official language preference. Requests for Technical Reports in the second official language should be sent to the address on the back of the title page.**

SÉRIE DE RAPPORTS TECHNIQUES
DU SERVICE CANADIEN DE LA FAUNE

Cette série de rapports donnant des informations scientifiques et techniques sur les projets du Service canadien de la faune (SCF) a démarré en 1986. L'objet de ces rapports est de promouvoir la diffusion d'études s'adressant à un public restreint ou trop volumineuses pour paraître dans une revue scientifique ou l'une des séries du SCF.

Ordinairement, seuls les spécialistes des sujets traités demandent ces rapports techniques. Ces documents ne sont donc produits qu'à l'échelon régional et en quantités limitées; ils ne peuvent être obtenus qu'à l'adresse figurant au dos de la page titre. Cependant, leur numérotage est effectué à l'échelle nationale. La citation recommandée apparaît à la page titre.

Ces rapports se trouvent dans les bibliothèques du SCF et figurent aussi dans la liste de la Bibliothèque nationale du Canada utilisée dans les principales bibliothèques scientifiques du Canada. Ils sont publiés dans la langue officielle choisie par l'auteur en fonction du public visé, avec un résumé dans la deuxième langue officielle. **En vue de déterminer si la demande est suffisamment importante pour produire ces rapports dans la deuxième langue officielle, le SCF invite les usagers à lui indiquer leur langue officielle préférée. Il faut envoyer les demandes de rapports techniques dans la deuxième langue officielle à l'adresse indiquée au verso de la page titre.**

USE OF THE COMET ASSAY TO ASSESS GENOTOXICITY IN MAMMALIAN, AVIAN, AND AMPHIBIAN SPECIES

L. D. Knopper, Ph.D.

National Wildlife Research Centre
Canadian Wildlife Service
Environment Canada

Technical Report Series No. 429
Headquarters 2005
Canadian Wildlife Service

Issued under the Authority of the
Minister of Environment
Canadian Wildlife Service

© Minister of Public Works and Government Services Canada 2005
Catalogue No. CW69-5/429E-PDF
ISBN 0-662-39436-4

This report may be cited as: Knopper, L.D. (2005) Use of the comet assay to assess genotoxicity in mammalian, avian, and amphibian species. Technical Report Series No. 429. Canadian Wildlife Service, Headquarters, Ottawa, ON, Canada.

PREFACE

The purpose of this Technical Report is to present background information and standard operating procedures (SOPs) for use of the Comet Assay (Single-Cell Gel Electrophoresis Test) to measure DNA damage (e.g.: double strand breaks, single strand breaks, alkali-labile sites expressed as single strand breaks) in mammalian, avian and amphibian species. The following SOPs have been conducted using cells from CD-1 mice, meadow voles (*Microtus pennsylvanicus*), chickens (*Gallus domesticus*), mallard ducks (*Anas platyrhynchos*), leopard frogs (*Rana pipiens*) and green frogs (*Rana clamitans*). It is probable that cells from other species can be used following these SOPs, but prior to experimentation, techniques will need to be validated.

The general comet assay technique is composed of seven steps: 1) dilution of the cells of interest, 2) mixing the cell suspension with agarose to create a microgel, 3) lysis of cell and nuclear membranes, 4) unwinding of exposed DNA, 5) electrophoresis, 6) neutralization of migrated DNA, 7) dehydration of gels. Dilution of cell suspensions, components and pH of buffers, and the voltage and time used for electrophoresis, are all dependent on the cell type being assessed. To quantify DNA damage, gels are stained with a DNA binding dye and individual cells are scored for DNA migration using a fluorescence microscope and computer software specific to the comet assay. Numerous metrics can be used to quantify levels of genotoxicity but comet tail length, percent DNA in the tail, and tail moment are the most commonly used endpoints.

The comet assay is a useful technique for biomonitoring studies because of its fairly inexpensive running costs (once major equipment is purchased), sensitivity, requirement for small numbers of cells, and rapid production of data. However, this technique is very sensitive to subtle changes that can yield appreciable differences in results, and data analysis is not without pitfalls. Researchers should be aware of these complexities by becoming familiar with the scientific literature prior to conducting experimental studies.

PRÉFACE

Ce rapport technique présente l'information de base ainsi que la procédure normale d'utilisation pour le test des comètes, lequel a pour but la détection des cassures de brin d'ADN chez les espèces de mammifères, d'oiseaux et d'amphibiens. La procédure décrite a été appliquée à des cellules de souris CD-1, de campagnol des champs (*Microtus pennsylvanicus*), de poulet (*Gallus domesticus*), de canard colvert (*Anas platyrhynchos*), de grenouille léopard (*Rana pipiens*) et grenouille verte (*Rana clamitans*). Des cellules d'autres espèces pourraient possiblement être utilisées, quoique les techniques devraient être validées au préalable.

La technique générale d'analyse des comètes comprend sept étapes: 1) diluer les cellules d'intérêt, 2) mélanger les cellules en suspension avec de l'agarose afin d'obtenir un microgel, 3) procéder à la lyse des cellules et des membranes nucléaires, 4) dérouler l'ADN exposé, 5) procéder à l'électrophorèse, 6) neutraliser l'ADN et 7) déshydrater les gels. La dilution cellulaire, la composition et le pH de la solution tampon, ainsi que le voltage et la durée de l'électrophorèse dépendent du type de cellules. Afin de quantifier le dommage à l'ADN, une teinture se liant à l'ADN est alors appliquée au gel et la migration de l'ADN est mesurée à l'aide d'un microscope à fluorescence et un programme informatique conçu à cet effet. Plusieurs mesures peuvent servir à quantifier la génotoxicité mais les mesures les plus couramment utilisées sont la longueur la queue de la comète, la proportion d'ADN dans la queue et le moment.

L'analyse des comètes est utile dans le cadre de contrôles biologiques étant donné que cette technique est assez peu dispendieuse (lorsque les équipements de base sont acquis), qu'elle est sensible, requiert peu de cellules et les données peuvent être générées rapidement. Cette technique est cependant très sensible à des changements subtils lesquels peuvent affecter les résultats de façon appréciable et l'analyse des données n'est pas sans difficultés. Les chercheurs doivent donc être au courant de ces complexités en se familiarisant avec la littérature scientifique avant d'effectuer leurs travaux.

TABLE OF CONTENTS

PREFACE.....i

PRÉFACE.....ii

TABLE OF CONTENTS.....iii

1. BACKGROUND INFORMATION.....1

1.1. DILUTIONS.....2

1.2. BUFFER PH.....2

1.3. VOLTAGE AND TIME OF ELECTROPHORESIS.....3

1.4. CALIBRATION.....4

1.5. CELL VIABILITY.....5

1.6. FREEZING SAMPLES.....6

1.7. SPECIES CONCERNS.....6

1.8. METRIC FOR ASSESSING DAMAGE.....7

1.9. FIGURE 1.....9

2. SOP FOR THE COMET ASSAY.....10

2.1. SAFETY PRECAUTIONS.....11

2.2. REAGENTS.....11

2.3. SOLUTIONS.....12

2.4. EQUIPMENT.....15

2.5. DETAILED PROCEDURE.....17

3. CITED AND USEFUL REFERENCES.....21

4. APPENDIX I.....27

1. Background Information

The comet assay, also known as the single-cell gel electrophoresis test, is a method of detecting DNA strand breakage (double, single, and alkali-labile sites expressed as single strand breaks) in virtually any nucleated cell. Significant advantages of the comet assay over other genotoxicity tests are its fairly straight forward technique, sensitivity, requirement for small numbers of cells (making the assay conducive to non-lethal testing) and rapid production of data (Tice et al. 2000). Saying this, researchers need to be aware that the technique is very sensitive to subtle changes that can yield appreciable differences in results, and data analysis is not without pitfalls. The purpose of this Technical Report is to present background information and SOPs for use of the comet assay in wildlife ecotoxicology. It should not be considered a formative training manual. Prior to conducting any experiment using the comet assay, researchers are encouraged to familiarize themselves with the scientific literature and software operating manuals.

The general comet assay technique starts by diluting nucleated cells of interest and mixing the cell suspension with liquefied low-melting point agarose. An aliquot of the cell-agarose suspension is cast into individual plastic chambers affixed to a piece of agarose gel support medium (e.g.: GelBond film)(McNamee et al. 2000). Some labs prefer to deposit the cell-agarose suspension in between layers of solid agarose on frosted glass slides, but the GelBond technique is without the main technical problems associated with using slides (e.g., lack of agarose adhesion to slides, shrinking of gels during long term storage). After the solidification of the cell-agarose suspension, gels are placed in a lysis buffer consisting of salts and detergents to expose the nucleus. This is followed by

submersion of gels in an alkaline or neutral buffer to allow for DNA unwinding prior to electrophoresis. After electrophoresis, gels are placed in a neutralizing buffer to fix the migrated DNA, and then dehydrated in ethanol. This technique is common to most researchers but cell dilutions, buffer pH, and voltage used and time of electrophoresis are specific to cell type and equipment being used.

1.1. *Dilutions*

Since the basis of the comet assay is to measure DNA damage in individual cells (i.e.: generally no fewer than 50 per sample), it is important that cell density in the gels is not too high otherwise tails of cells may overlap with heads of other cells, making measurements impractical. Generally, a dilution of a sample resulting in 2 x 10^4 or 10^5 cells / ml is sufficient. For example, mammalian whole blood requires a 1/10 dilution with phosphate buffered saline (PBS) whereas amphibian whole blood requires a 1/100 dilution.

1.2. *Buffer pH*

Under neutral pH conditions, only double stranded DNA breaks can be revealed using the comet assay (Olive et al. 1991). Under alkaline pH conditions (> 13.1), double and single strand breaks as well as alkali-labile sites (expressed as single strand breaks) can be detected (Tice et al. 2000; Hartmann et al. 2003). Compared to radiation exposure, chemical genotoxins cause orders of magnitude fewer double strand breaks than single strand breaks (Tice et al. 2000). Thus, the alkaline comet

assay is a more useful tool for biomonitoring studies than the neutral version. However, in some cases cells (e.g.: sperm) may contain inherently high levels of ambient alkali-labile sites (Singh et al. 1989). Alkaline buffers will not be useful when using these cell types because these sites will be expressed as single strand breaks. Thus, control cells will exhibit substantial DNA damage. In these circumstances the use of neutral buffers is suggested (e.g.: TBE; pH 8.2). Some researchers have used electrophoresis buffers of pH 9.0 (Duty et al. 2003) and 12.5 (Migliore et al. 2002) with no apparent increases in ambient DNA damage, but others have not been able to reproduce these results in controlled laboratory experiments (Knopper, unpublished data).

1.3. *Voltage and Time of Electrophoresis*

If the voltage or running time used in electrophoresis is too low, DNA will not migrate from cells. Conversely, if the voltage or running time is too high, DNA from undamaged cells will be pulled apart and cells will exhibit the comet shape associated with damage. Thus, conditions need to be optimized so only true damage is detected. Generally, control cells should exhibit no more than 5-10 % DNA in the tail and have tail lengths of no more than 15-20 μm (Collins 2004). The appropriate voltage and time of electrophoresis will vary depending on the species and cell type being assessed, the equipment being used, and on the volume of buffer required to cover the gels. As a general practice, gels should be covered with approximately 1 cm of buffer. It is very important to be consistent with the volume of buffer being used

in the electrophoresis units because different volumes will result in different patterns of DNA migration. This is based on the equation V= IR, where V is volts, I is current (milliamps) and R is resistance. Voltages (constant voltage) should be expressed as V/cm, the distance between the anode and cathode in the electrophoresis units. A good starting point for blood samples is 0.7 V/cm for between 16 and 20 minutes, and 0.4 V/cm for 20 minutes for sperm. It is imperative that these conditions be optimized before an experiment is conducted and then adhered to throughout the experimental procedure.

1.4. *Calibration*

Calibration of the comet assay technique can be achieved by assessing DNA damage in cells that have had strand breaks induced via radiation or chemical exposure. Generating a dose-response curve can also be used to assess the sensitivity of the technique. Calibration should be conducted with each new species and cell type being assessed.

Unless a Gamma Cell radiation machine is available for use, exposure of cells to hydrogen peroxide (H2O2), cyclophosphamide, or methyl methanesulfonate is an acceptable method of generating DNA strand breaks for positive controls (Tice et al. 2000; Hartmann et al. 2003; Singh et al. 2003). In the case of germ cells, the chromatin is very tightly packed, so cells must undergo lysis prior to chemical exposure. This is not necessary if DNA damage is being induced by radiation exposure (Singh et al. 2003).

1.5. *Cell viability*

Cells that are apoptotic and necrotic do not display the typical comet appearance but exhibit very small or non-existent heads and very large diffuse tails. These cells are commonly referred to as tear-drops, ghost cells or hedgehogs cells (Tice et al. 2000; Hartmann et al. 2003). Such cells can be produced after exposure to cytotoxic, non-genotoxic agents, and should not be measured in data analysis. Cells that have been exposed to genotoxins can also show this type of appearance and should be used in data analysis. Thus, it is important to conduct a concomitant assessment of cytotoxicity in the cell suspension in order to determine the cause of highly damaged cells.

Cell viability is predominantly assessed using one of two methods. The first is with the dual stain viability assay (Strauss 1991). Here cells are mixed with ethidium bromide and fluorescein diacetate and viewed in a hemacytometer under fluorescence microscopy. Viable cells fluoresce green and non-viable cells fluoresce red. Another way to assess viability is by adding Trypan blue to the cell suspension (equal parts Trypan blue and cell suspension; e.g.: 5 µl Trypan blue plus 5 µl diluted cell suspension). Cells that take up the dye are nonviable, whereas those that exclude the dye are viable. In general, samples with viability below 70-75% of that in the control samples should be discarded from further analysis (Tice et al. 2000).

1.6. *Freezing samples*

When possible, fresh samples should be used for the comet assay. When this is not possible, samples can be frozen in a cryopreservant (PBS and 10% dimethyl sulfoxide (DMSO)), and flash frozen in liquid nitrogen. Samples can be stored at -80°C for convenience. If using cells from tissues, tissue should be minced and then handled the same way. Whole tissue should not be frozen as this will result in increased ambient DNA damage due to cell swelling (personal communication, M. Vasquez, Helix3, Inc., Research Triangle Park, NC). In some labs, researchers prefer to freeze samples in the vapour phase of liquid nitrogen prior to full submersion and some advocate adding ethylenediaminetetraacetic acid (EDTA) to the cryopreservant to prevent potential endonuclease activity.

1.7. *Species concerns*

The comet assay can be conducted using virtually any nucleated cell, with the caveat that those cells are viable. It has been found that in some species with nucleated erythrocytes, not all of the cell population is viable. For example, in studies with undosed chickens and ducks (unpublished data, Stoddart, R. University of Saskatoon, Saskatoon, SK, Canada) and great blue herons (personal communication, Steinert, S.A., CSC Biomarker Laboratory, San Diego, CA, USA) it has been found that whole blood is not appropriate for use with the comet assay because over 80% of the cells exhibit the "ghost cell" or "hedgehog" appearance. In

these cases, leukocytes need to be used for the comet assay (refer to Appendix I for a SOP for leukocyte separation). Whole blood from amphibians does not appear to display this type of cell segregation.

1.8. *Metrics for assessing damage*

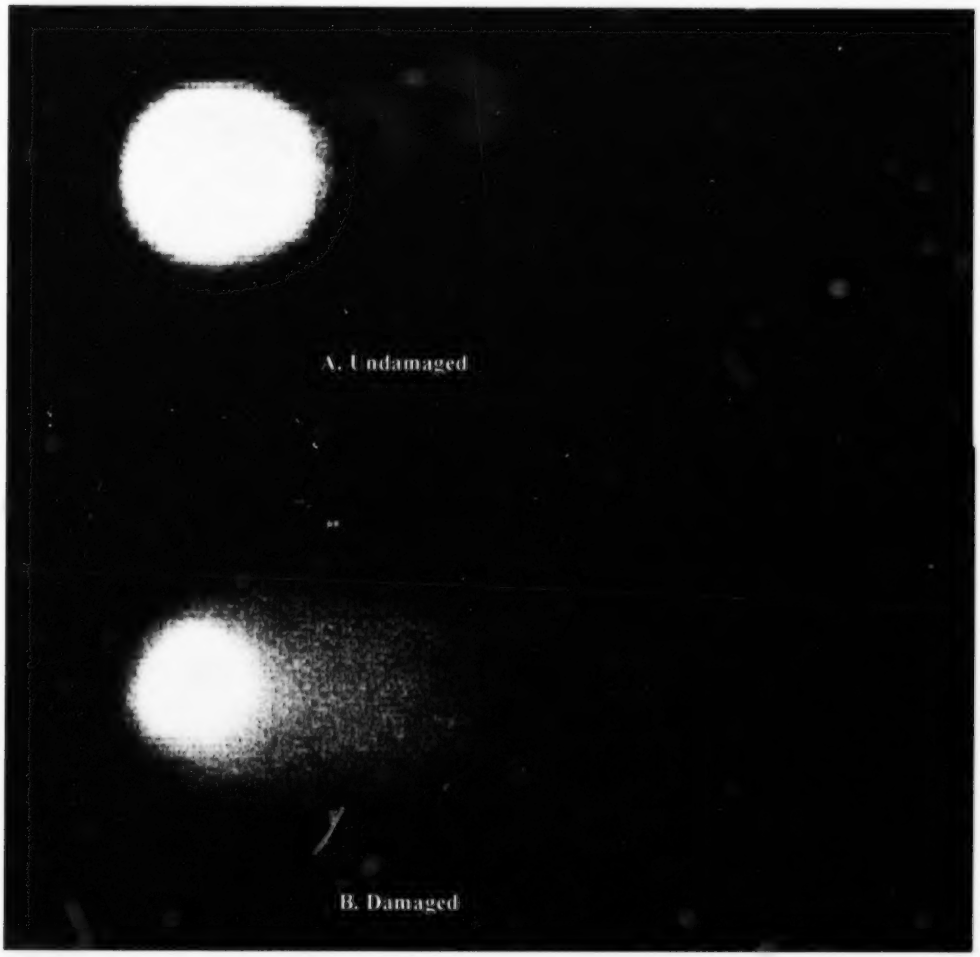
To quantify DNA damage, gels are stained with a DNA stain (e.g.: ethidium bromide, SYBR Gold, SYBR Green) and the cells scored for DNA migration under a fluorescence microscope using an appropriate software package (e.g.: Komet (Kinetic Imaging, Nottingham, UK), Comet Assay (Perceptive Instruments, Suffolk, UK)). Damaged cells have an appearance similar to astronomical comets, with long tails of DNA migrating from the center of the exposed nucleoid (Figure 1). Damage is generally quantified using three main values: comet tail length, tail moment (i.e.: tail length multiplied by the %DNA in the tail) or Olive tail moment (i.e.: the centre of gravity of the tail minus the centre of gravity of the head all multiplied by %DNA in the tail), and %DNA in the tail (Collins et al. 1997; Tice et al. 2000). The most informative metric is currently under debate by the comet assay community, mainly because computerized imaging programs tend to compute these metrics differently.

Tail length is expected to increase quickly with low levels of exposure to a genotoxin but plateaus at higher exposures (Fairbairn et al. 1995) because DNA can only migrate so far due to electrophoretic conditions. However, the amount of DNA in the tail region can continue to increase as the dose increases, theoretically from 0 to 100%. Thus, with increasing dose, it is tail intensity that increases, not tail length

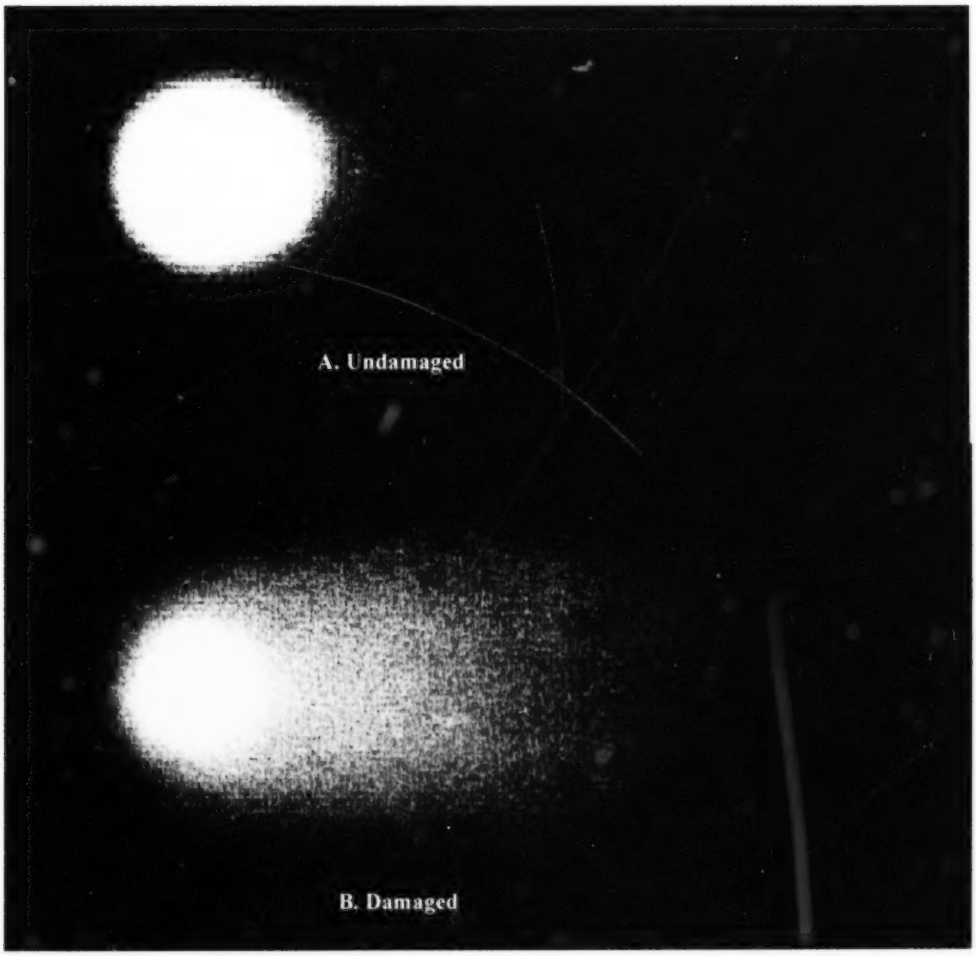
(Collins et al. 1997). Since moment is calculated based on length, it has been argued that tail intensity, or percent DNA in the tail, should be the best metric of genotoxicity (Collins 2004). In relation to data obtained from time-course biomonitoring studies, tail length can be used to indicate initial DNA damage and confirm exposure to a genotoxin, while tail moment and percent DNA in the tail can be used to indicate the intensity of damage (Knopper et al. in press).

The National Institute of Health maintains a Listserve for the Comet Assay Interest Group (www.cometassay.com), where ongoing discussion and debate takes place regarding all aspects of the comet assay. The Listserve is regularly updated and all correspondence is archived so past postings can be viewed.

1.9. FIGURE 1. Image of an alkaline comet (leukocyte) taken with a 40X oil immersion objective lens (total magnification 330X) stained with SYBR Gold stain. A) Undamaged cell, B) damaged cell.



1.9. FIGURE 1. Image of an alkaline comet (leukocyte) taken with a 40X oil immersion objective lens (total magnification 330X) stained with SYBR Gold stain. A) Undamaged cell, B) damaged cell.



2. STANDARD OPERATING PROCEDURE FOR THE COMET ASSAY

The names of manufacturers and suppliers are included in the following SOPs only to document the exact assay conditions adopted at the National Wildlife Research Centre (NWRC), Canadian Wildlife Service, Environment Canada, Ottawa. Other equivalent products, instruments or reagents from other sources may also give satisfactory results.

2.1. SAFETY PRECAUTIONS

- General safety rules and waste disposal procedures that apply to laboratory work must be followed.
- Adequate protective equipment must be used: lab coat, gloves, mask (when preparing solutions and tissues), cryogloves and face shield or goggles (when applicable).
- Material Safety Data Sheets (MSDS) for the use and disposal of products used in the comet assay must be read and understood.

2.2. REAGENTS

- Sodium chloride, NaCl; Sigma # S6191
- Potassium phosphate monobasic, KH2PO4; Sigma # P9791
- Sodium Phosphate dibasic, Na2HPO4 ; Sigma # 71643
- Agarose, Low melting point; Sigma #A9419
- Triton X-100, Sigma # T9284
- Agarose-1000; Sigma # A9539
- Ethylenediaminetetraacetic acid tetrasodium salt dehydrate (EDTA); Sigma # ED4SS
- N-Lauroylsarcosine sodium salt; Sigma # L9150
- Sodium Hydroxide, NaOH; Sigma #S-0899
- 8-hydroxyquinoline; Sigma #252565

- Ammonium acetate; Sigma #A1542
- Trizma base; Sigma # T6791
- Tris-Hydrochloride; Sigma # T3253
- Boric Acid; Sigma #B-1934
- Dimethyl Sulfoxide, DMSO; Sigma D-5879
- Hydrogen Peroxide (30%); Sigma # H-1009
- Ethanol; Sigma # 459836
- Fluorescein diacetate; Sigma #F-7378
- DL- Dithiothreitol (DTT); Sigma # D5545
- SYBR Gold nucleic acid gel stain, Molecular Probes #S-11494
- Ethidium bromide, GibcoBRL # 15585
- Trypan blue; VWR # VW8721-0
- Proteinase K; Amresco # 0706
- Purified water from the Milli-RO / Milli-Q system.

2.3. SOLUTIONS

- 10X Ca^{++} and Mg^{++} free PBS:

  1.31 M NaCl = 76.5 g
  50 mM Na_2HPO_4 = 7.25 g
  16 mM KH_2PO_4 = 2.12 g

  Add approximately 900 ml purified H_2O. Bring to a volume of 1 L.
  Dilute to 1X before use and adjust to pH 7.4.

- 0.75% Agarose:

Low melting point (LMP) agarose = 750 mg
Phosphate buffered saline (PBS) = 100 ml

Stir together on stirring plate with heat on medium/high and then dispense 1.0 ml aliquots into 1.5 ml Eppendorf tubes. Store at 4°C until needed. On the day of experiment, melt in microwave for 10-20 seconds on high and place in heater block at 42°C.

- 1.0% Agarose:

  Agarose = 1.0 g
  Phosphate buffered saline (PBS) = 100 ml

  Stir together on stirring plate with heat on medium/high and then dispense 4 ml aliquots into Falcon tubes and store at 4°C until use. On the day of experiment, melt in microwave for 10-20 seconds on high.

- Lysis Buffer A (blood for mammalian, avian and amphibian species):

  2.5M NaCl = 146.1 g/L
  100mM Tetra-sodium EDTA = 41.6 g/L
  10mM Tris base = 1.2 g/L
  1% N-Lauryl Sarcosine = 10.0 g/L

  Cover and shake to remove clumps. Slowly add 1 L purified H_20 and stir on a stir plate. Adjust pH to 10.0. Store in the dark at room temperature. **Note: Add 1% Triton X-100 to required volume on the day of experiment, 30 minutes prior to use. Store at 4°C (see below).**

- Lysis Buffer B (sperm for mammalian species):

  2.5M NaCl = 146.1 g/L
  100 mM Tetra-sodium EDTA = 41.6 g/L
  10.0 mM Tris HCL = 0.61 g/L

  Cover and shake to remove clumps. Slowly add 1 L purified H_20 and stir on a stir plate. Adjust pH to 10.0. Store in the dark at room temperature. **Note: Add 1% Triton X-100 and 4mM DTT to required volume on the day of experiment and stir. DO NOT refrigerate. Add 0.1 mg/ml PK when required (see below).**

- Electrophoresis Buffer A (blood for mammalian, avian and amphibian species):

  0.3 M NaOH = 12.0 g/L

10 mM Tetra-sodium EDTA = 4.2 g/L
0.1% 8-hydroxyquinoline = 1.0 g/L

Slowly add 980 purified H_20 and stir on a stir plate. Add 2% (v/v) DMSO while mixing. Adjust to pH 13.1 with concentrated NaOH or HCl. Prepare fresh on day of experiment.

- Electrophoresis Buffer B (sperm for mammalian species):

  *For 1L of **10X** TBE buffer:*

  0.1M Tris = 121.2 g/L
  0.09M Boric Acid = 55.6 g/L
  0.001M EDTA = 4.2 g/L

  Slowly add 1 L of purified H_20 and stir on stir plate. Adjust pH to 8.2 with concentrated NaOH or HCl.

- Neutralization Buffer:

  1 M Ammonium acetate = 7.7 g

  Slowly add 100 ml purified H_20 and stir on stir plate. Adjust pH to 7.0 with concentrated NaOH or HCl.

- Dual stain for cell viability:

  stock fluorescein diacetate (5.0 mg / ml acetone)
  stock ethidium bromide (200 µg / ml PBS)—pH 7.4

  Store stock fluorescein diacetate at – 20°C and stock ethidium bromide, wrapped with aluminum foil, at 4°C.

  In an Eppendorf tube add:

  1.2 ml PBS
  7.5 µl stock fluorescein diacetate
  50 µl stock ethidium bromide

  Make fresh on day of use.

- DNA Stain:

  SYBR Gold stain = 5.0 µl

Thaw SYBR Gold. Take a 5.0 µl aliquot and dispense into 50 ml purified H_2O. Wrap in aluminum foil. Store in the dark at 4°C. When sensitivity has diminished, another 5.0 µl of SYBR Gold can be added to replenish the solution. When this needs replenishing, new stock should be made. Dispose of solution by pouring through activated charcoal.

2.4. EQUIPMENT

2.4.1. Glassware and Labware

- Pipettes
- Pipette tips (10, 20, 100, 200, 1000 µl)
- Volumetric flasks (100 ml, 500 ml, and 1 L)
- Graduated cylinders (100 ml, 500 ml, 1 L)
- Beakers (250 ml, 500 ml, 1 L)
- Petri dishes
- Dispensing bottle (for purified water)
- Ice bucket
- Cryovials (1.0 ml)
- Eppendorf tubes (0.5 ml, 1.5 ml)
- Centrifuge tubes with screw caps (50 ml)
- Plastic/glass trays (e.g., lids and bottoms of pipette tips containers)
- Microscope slides
- Microscope cover slips (22 x 50mm)

2.4.2. Instrumentation

- pH meter
- Analytical balance
- Magnetic stirrer and magnetic bars
- Power source (capable of low voltage and high milliamperage, e.g.; 300V, 2000 mA)
- Horizontal gel electrophoresis units
- Tweezers
- Scissors
- GelBond Film (agarose gel support medium); Mandel # 53740 GB 1638
- Lab Tek II chambers (Nalge Nunc #154461-B)
- Heater block
- Computer with Comet assay imaging system (e.g., Kinetic Komet 5.5 software)
- Fluorescence microscope with 40x oil immersion objective (e.g., Zeiss AxioPlan II) and proper excitation/emission filters for dye (e.g., SYBR Gold: ex 300, em 495/537 nm, bound to nucleic acid)

2.5. DETAILED PROCEDURE

- Remove 1.5 ml eppendorf tubes of 0.75% low melting point agarose (LMP) and 1% agarose from the fridge and melt in microwave for approximately 10-20 seconds on high.
- Once melted, place tubes in a pre-warmed heat block set at 42°C.
- Pour the 1% agarose in small Petri dish.
- Gently dip a gloved finger in the melted agarose and run along the bottom of a Lab-Tek II chamber until bottom is coated (New Lab-Tek II chambers have an adhesive and do not need to have agarose applied until the adhesive wears off).
- Affix Lab-Tek II chambers to GelBond by applying constant pressure for approximately 30 seconds.

ALL REMAINING STEPS SHOULD BE CONDUCTED UNDER SUBDUED LIGHTING

- If using frozen samples: Remove samples from -80°C freezer and place in room temperature water bath. Once the samples have thawed (~1-3 minutes), gently mix and place samples on ice.
- From the diluted cell suspensions (depending on sample, see above) take a 30 µl aliquot and add to 270 µl of liquefied 0.75% LMP agarose. Mix gently by pipetting.

- Cast a 120 µl aliquot of the cell/agarose mixture into an individual well of the 2-well chamber.
- Cast another 120 µl aliquot into a different chamber affixed to a different GelBond film. Repeat with each blood sample (This duplicate gel should be stored as a backup in case the other is damaged).
- An internal control should be run simultaneously. If available, use a control sample. If no control is available, human blood (dilution: 20 µl of human blood mixed with 180 µl of PBS), obtained from finger prick, is a suitable substitute. Control samples can be frozen ahead of time and thawed when needed, but the viability and DNA damage in these samples should be checked regularly for consistency as time spent frozen is related to increased DNA damage.
- Once the agarose has solidified (approximately 3 minutes), carefully remove the Lab-Tek II chambers and place each GelBond film in a small plastic box filled with 75 ml Lysis Buffer (Lysis A for leukocytes, Lysis B for germ cells).
- Place dishes, covered with aluminum foil, in the fridge (4°C) overnight.
- For sperm: Place GelBond with agarose in Lysis B containing dithiotreitol (DTT) for 1 hour. From here, place GelBond with agarose in Lysis B containing proteinase K and place at 37°C overnight (either in an incubator or, because of the odor associated with DTT, in a warm water bath inside a fume hood).
- The following day, calibrate pH meter (follow instruction in pH meter booklet).
- Make fresh Electrophoresis Buffer (leukocytes: pH 13.1; germ cells: pH 8.2 or 12.1).

- Remove gels from fridge/incubator/water bath.
- Place a Petri dish in the sink and fill with fresh water. Allow water to gently run into dish.
- Using tweezers, remove GelBond from Lysis buffer and repeatedly dip into the Petri dish for about 30 seconds, or until the foam from the Lysis buffer has drained. Salt from Lysis buffer that remains on the gel can cause misshapen comets during electrophoresis so proper rinsing should be ensured.
- Place GelBond film into the gel electrophoresis units filled with 150 ml of Electrophoresis Buffer. Let gels sit for 30 minutes.
- After 30 minutes, electrophorese gels using constant voltage (time and voltage will depend on sample type).
- Place a Petri dish in the sink and fill with fresh water. Allow water to gently run into dish.
- Using tweezers, gently remove the GelBond film from the electrophoresis unit and repeatedly dip into the Petri dish for about 30 seconds, or until the foam from the buffer has drained.
- Transfer GelBond film to another tray containing 75 ml of Neutralization buffer and leave for 30 minutes.
- After 30 minutes, rinse as above, and place GelBond film into ~75 ml of 85% ethanol for a minimum of 2 hours.
- Remove film and then air-dry by hanging overnight.
- Store dried gels in labeled manila envelopes.

- To stain gels, make SYBR Gold solution in a 50 ml tube (see 1.2.10) and fill another 50 ml tube with water.
- Cut one of the replicate GelBonds into strips, so each strip contains 2 gels.
- Label with a cryopen (permanent marker seems to fade in the dye), and place in stain for 10-30 minutes (leukocytes from mammals and birds and mammalian sperm needs between 10-15 minutes staining; whole blood from amphibians needs 30 minutes).
- After the staining period, remove GelBond strip with tweezers, and dip in water two or three times.
- Place GelBond, gel side up, on microscope slide and cover with a 22 x 50 mm glass cover slip.
- Gently press with a paper towel to remove excess water and to form a seal.
- Place on microscope with immersion oil and score a minimum of 50 cells using appropriate software.

3. CITED AND USEFUL REFERENCES

1. Anderson, D., Yu, T-W., Dobrzynska, M. M., Ribas, G. and Marcos, R. (1997). Effects in the comet assay of storage conditions on human blood. Teratogenesis, Carcinogenesis, and Mutagenesis 17: 115-125.

2. Anderson, D., Dobrzynska, M. M., Jackson, L. I., Yu, T-W., and Brinkworth, M. H. (1997). Somatic and germ cell effects in rats and mice after treatment with 1,3-butadiene and its metabolites, 1,2-epoxybutene and 1,2,3,4-diepoxybutane. Mutation Research 391: 233-242.

3. Anderson, D., Dobrzynska, M. M., and Basaran, N. (1997). Effect of various genotoxins and reproductive toxins in human lymphocytes and sperm in the comet assay. Teratogenesis, Carcinogenesis, and Mutagenesis 17: 29-43.

4. Clements, C., Ralph, S., and Petras, M. (1997). Genotoxicity of select herbicides in *Rana catesbeiana* tadpoles using alkaline single-cell gel DNA electrophoresis (comet) assay. Environmental and Molecular Mutagenesis 29: 277-288.

5. Collins, A. R., Dobson, V. L., Dusinská, M., Kennedy, G., and Stetina, R. (1997). The comet assay: what can it really tell us? Mutation Research 375: 183-193.

6. Collins, A. R. (2004). The Comet Assay for DNA Damage and Repair: Principles, Applications, and Limitations. Molecular Biotechnology 26: 249-261.

7. Cotelle, S., and Férard, J. F. (1999). Comet assay in genetic ecotoxicology: a review. Environmental and Molecular Mutagenesis 34: 246-255.

8. Duty, S. M., Singh, N. P., Ryan, L., Chen, Z., Lewis, C., Huang, T., and Hauser, R. (2002). Reliability of the comet assay in crypopreserved human sperm. Human Reproduction 17: 1274-1280.

9. Duty, S. M., Singh, N. P., Silva, M., Barr, D. B., Brock, J. W., Ryan, L., Herrick, R. F., Christiani, D. C., and Hauser, R. (2003). The relationship between environmental exposures to phthalates and DNA damage in human sperm using the neutral comet assay. Environmental Health Perspectives 111: 1164-1169.

10. Fairbairn, D. W., Olive, P. L., and O'Neil, K. L. (1995). The comet assay: a comprehensive review. Mutation Research 339: 37-59.

11. Garaj-Vrhovac, V., and Zeljezic, D. (2000). Evaluation of DNA damage in workers occupationally exposed to pesticides using single-cell gel electrophoresis (SCGE) assay-pesticide genotoxicity revealed by comet assay. Mutation Research 469: 279-285.

12. Godard, T., Fessard, V., Huet, S., Mourot, A., Deslandes, E., Pottier, D., Hyrien, O., Sichel, F., Gauduchon, P., and Poul, J. M. (1999). Comparative in vitro and in vivo assessment of genotoxic effects of etoposide and chlorothalonil by the comet assay. Mutation Research 444: 103-116.

13. Haines, G.A., Hendry, J.H., Daniel, C.P., and Morris, I.D. (2002). Germ cell and dose-dependent DNA damage measured by the comet assay in Murine spermatozoaa after testicular x-irradiation. Biology of Reproduction 67: 854-861.

14. Hartmann, A., Agurell, E., Beevers, C., Brendler-Schwaab, S., Burlinson, B., Clay, P., Collins, A. R., Smith, A., Speit, G., Thybaud, V., and Tice, R. R. (2003). Recommendations for conducting the in vivo alkaline comet assay. Mutagenesis 118: 45-51.

15. Heuser, V., da Silva, J., Moriske, H-J., Dias, J. F., Yoneama, M. L., and de Freitas, T. R. O. (2002). Genotoxicity biomonitoring in regions exposed to vehicle emissions using the comet assay and the micronucleus test in native rodent *Ctenomys minutus*. Environmental and Molecular Mutagenesis 40, 227-235.

16. Knopper, L. D., Mineau, P., McNamee, J. P., and Lean, D. R. S. (In Press) Effects of pesticide exposure on meadow voles (*Microtus pennsylvanicus*) living in golf course ecosystems: Genotoxicity measured with the comet and micronucleus assay, Ecotoxicology.

17. Krause, G., Wolz, L., and Scherer, G. (2001). Performing the "comet assay" for genetic toxicology applications. Life Sciences News #7, Amersham Pharmacia Biotech, Piscataway, NJ, USA.

18. Lebailly, P., Vigreux, C., Godard, T., Sichel, F., Bar, E., LeTalaër, J. Y., Henry-Amar, M., and Gauduchon, P. (1997). Assessment of DNA damage induced in vitro by etoposide and two fungicides (carbendazim and chlorothalonil) in human lymphocytes with the comet assay. Mutation Research 375: 205-217.

19. Lee, R. F. and Steinert, S. (2003). Use of the single cell electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. Mutation Research 544, 43-64.

20. McNamee, J. P., McLean, J. R. N., Ferrarotto, C. and Bellier, P. V. (2000). Comet assay: rapid processing of multiple samples. Mutation Research 466: 63-69.

21. Migliore, L., Naccarati, A, Zanello, A, Scarpato, R., Bramanti, L., and Mariani, M. (2002). Assessment of sperm DNA integrity in workers exposed to styrene. Human Reproduction 17: 2912-2918.

22. Morris, I. D., Ilott, S., Dixon, L., and Brison, D. R. (2002). The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (comet assay) and its relationship to fertilization and embryo development. Human Reproduction 17: 990-998.

23. Olive, P. L., Wlodek, D., and Banáth, J. P. (1991). DNA double-strand breaks measured in individual cells subjected to gel electrophoresis. Cancer Research 51: 4671–4676.

24. Olive, P. L. (1999). DNA damage and repair in individual cells: applications of the comet assay in radiobiology. International Journal of Radiation Biology 75: 395-405.

25. Ralph, S., Petras, M., Pandrangi, R., and Vrzoc, M. (1996). Alkaline single-cell gel (comet) assay and genotoxicity monitoring using two species of tadpoles. Environmental and Molecular Mutagenesis 28: 112-120.

26. Ralph, S., and Petras, M. (1997). Genotoxicity monitoring of small bodies of water using two species of tadpoles and the alkaline single cell gel (comet) assay. Environmental and Molecular Mutagenesis 29: 418-430.

27. Ribas, G., Frenzilli, G., Barale, R. and Marcos, R. (1995). Herbicide-induced DNA damage in human lymphocytes evaluated by the single-cell gel electrophoresis (SCGE) assay. Mutation Research 344: 41-54.

28. Schnurstein, A. and Braunbeck, T. (2001) Tail Moment versus Tail Length: Application of an in vitro version of the comet assay in biomonitoring for genotoxicity in native surface waters using primary hepatocytes and gill cells from zebrafish (*Danio rerio*). Ecotoxicology and Environmental Safety 49: 187-196.

29. Silva, J., de Freitas, T. R. O., Marinho, J. R., Speit, G., and Erdtmann, B. (2000). An alkaline single-cell gel electrophoresis (comet) assay for environmental biomonitoring with native rodents. Genetics and Molecular Biology 23: 241-245.

30. Singh, N. P., Danner, D. B., Tice, R. R., McCoy, M. T., Collins, G. D., and Schneider, E. L. (1989). Abundant alkali-sensitive sites in DNA of human and mouse sperm. Experimental Cell Research 184: 461-470.

31. Singh, N. P., Muller, C. H., and Berger, R. E. (2003). Effects of age on DNA double-strand breaks and apoptosis in human sperm. Fertility and Sterility 80: 1420-1430.

32. Speit, G., and Hartmann, A. (1999). The comet assay (single-cell gel test): A sensitive genotoxicity test for the detection of DNA damage and repair. In: Henderson DS (ed) Methods in Molecular Biology, pp 203-212. Humana Press, Totowa, NJ, USA.

33. Tice, R. R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J-C., and Sasaki, Y. F. (2000). Single cell gel/comet assay: Guidelines for the in vitro and in vivo genetic toxicity testing. Environmental and Molecular Mutagenesis 35: 206-221.

APPENDIX I

METHOD FOR THE SEPARATION OF AVIAN LYMPHOCYTES FROM WHOLE BLOOD USING FICOLL-PAQUE (MODIFIED VERSION PUBLISHED BY PHARMACIA BIOTECH)

1.0. Safety Precautions

- General safety rules and waste disposal procedures that apply to laboratory work must be followed.
- Adequate protective equipment must be used: lab coat, gloves, mask (when preparing solutions and tissues), cryogloves and face shield or goggles (when applicable).
- Material Safety Data Sheets (MSDS) for the use and disposal of products used in the comet assay must be read and understood.

2.0. Reagents

- Ficoll-Paque Plus; Pharmacia Biotech # 17-1440-02
- PBS
- Trypan blue; VWR # VW8721-0 or
- Ethidium bromide, GibcoBRL # 15585
- Fluorescein diacetate; Sigma #F-7378

3.0. Equipment

3.1.0. Glassware and Labware

- 15 ml blue-topped conical tubes
- Microcentrifuge tubes
- 20 gauge needles and 30cc syringe (for Ficoll-Paque)
- Pasteur pipettes

- Pipettes
- Pipette tips (10, 20, 100, 200, 1000 µl)
- Hemacytometer
- Cell counter
- Light/fluorescence microscope
- Centrifuge (preferably swinging-bucket style)

4.0. SEPARATION

- Label 3 tubes for each sample
- Add 250 µl PBS to tube #1 (1:1 with blood)
- Add 3.0 ml Ficoll-Paque to each tube #2 (using syringe and clean needle)
- Add 1.0 ml PBS to tube #3 (need >3x blood volume here)
- Store overnight at 4°C.

- To tube #1 add 250 µl blood (collected in heparinized capillary tube or syringe).
- Using a Pasteur pipette, mix contents of tube #1 (withdraw and expel once with pipette).
- Withdraw blood mixture from tube #1 and expel on top of Ficoll-Paque in tube #2 (Keep tube and pipette at 45°C and place tip on bottom inside of tube about ¼" from Ficoll-Paque).
- Balance tubes for centrifugation. Spin at 2000 rpm for 30 minutes.

- Using a Pasteur pipette, CAREFULLY withdraw WBC layer (buffy coat: looks like mist or snowflakes) and add to tube #3 (containing PBS).
- Mix cells in tube #3—might be slightly cloudy.
- Balance tubes for centrifugation. Spin at 2000 rpm for 10 minutes.
    - a) If centrifuge is fixed angle, then use Pasteur pipette to CAREFULLY remove as much supernatant as possible (maybe half) without disturbing WBCs at side of tube.
    - Discard supernatant, **OR**
    - b) If WBCs are pelleted at the bottom, invert the tube to pour off the supernatant.
- Top up the suspension in tube #3 with 500 µl PBS (you can use more or less but must record the volume in order to calculate the dilution factor) and resuspend cells by mixing.
- Store on ice.
- To determine cell volume, add 50 µl of WBC suspension to either trypan blue (50 µl) or to the dual stain mix, as in the cell viability assay technique for the comet assay, and gently mix.
- Load cell suspension into hemacytometer and count WBC. Dilute cell suspension to achieve approximately 2×10^4 or 10^5 cells / ml.